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A two-state stabilization-change mechanism for proton-pumping complex I ☆

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ABSTRACT

Despite its central function in oxidative phosphorylation, the molecular mechanism of proton pumping respiratory complex I is still elusive. In recent years, considerable progress has been made towards understanding structure/function relationships in this very large and complicated membrane protein complex. Last year X-ray crystallographic analysis of bacterial and mitochondrial complex I provided important insights into its molecular architecture. Based on this evidence, here a hypothetical molecular mechanism for redox-driven proton pumping of complex I is proposed. According to this mechanism, two pump modules are driven by two conformational strokes that are generated by stabilization of the anionic forms of semiquinone and ubiquinol that are formed in the peripheral arm of complex I during turnover. This results in the experimentally determined pumping stoichiometry of 4 $H^+/2e^-$. In the two-state model, electron transfer from iron–sulfur cluster N2 is allowed only in the 'E-state,' while protonation of the substrate is only possible in the stabilizing 'P-state.' In the membrane arm, transition from the E- to the P-state drives the two pump modules via long range conformational energy transfer through the recently discovered helical transmission element connecting them. The proposed two-state stabilization-change mechanism is fully reversible and thus inherently explains the operation of complex I in forward and reverse mode. This article is part of a Special Issue entitled Allosteric cooperativity in respiratory proteins.

1. Introduction

Reduction equivalents delivered via NADH to the respiratory chain of aerobic bacteria and mitochondria can be oxidized by different NADH-dehydrogenases that transfer the electrons onto ubiquinone or menaquinone [1]. One variant of these NADH-dehydrogenases that links this reaction to the vectorial transfer of protons across the bioenergetic membrane is commonly called respiratory chain complex I [2]. The pumping stoichiometry of complex I is considered to be 4 $H^+/2e^-$, but it should be noted that this value has been measured only for the mitochondrial enzyme that uses ubiquinone [3–5]. Therefore, the mechanism proposed here primarily applies for this substrate and modifications to the scheme may be necessary, in particular for menaquinone dependent enzymes. In bacteria, the L-shaped membrane integral enzyme complex typically consists of 14 different subunits with a total mass of about 550 kDa. It harbors one FMN and 8–9 iron–sulfur clusters as redox-prosthetic groups [6,7]. In addition to these central subunits, the mitochondrial version of complex I contains up to 34 accessory subunits resulting in a total mass of almost 1 MDa [8–11]. The reasons for the enormous size and complexity of this redox-driven proton pump remain obscure, but they provide an explanation for the fact that the detailed structure and molecular mechanism of complex I are still unknown. During some 50 years of research on complex I a remarkable number of mechanistic schemes have been proposed [12–14]. However, the recent insights into the molecular architecture of complex I by X-ray crystallographic analysis [15,16] render most of these models obsolete. Thus to stimulate future work, it seems timely to summarize the considerable advances complex I research has made, to highlight the open and controversial issues remaining and to compile our current knowledge into a hypothetical model.

2. Modules of complex I

Phylogenetic analysis of the origin of complex I revealed that its building blocks have evolved from different lineages of water soluble and membrane bound hydrogenases [17–20]. This resulted in a modular architecture [2,16] that is both functionally and structurally evident (Fig. 1). The N-module, for NADH-oxidation, is related to NAD^+-reducing hydrogenases like the one from *Alcaligenes eutrophus*. The Q-module, for quinone reduction, is most similar to the membrane bound NiFe-hydrogenases like the one from *Methanosarcina barkeri*. The P-module, for proton-pumping, contains three central subunits that are homologous to Mrp-type Na^+/H^+ antiporters found for example in *Bacillus subtilis* [21]. It is remarkable that these transporter-

Abbreviations: EPR, electron paramagnetic resonance; FMN, Flavine-mononucleotide; Q, $Q^{\cdot -}$,$QH^{\cdot}$, QH^-,QH_2, intermediates of ubiquinone; SQ_{Ns}, SQ_{Nf}, EPR detectable ubisemiquinone species bound to complex I

☆ This article is part of a Special Issue entitled Allosteric cooperativity in respiratory proteins.

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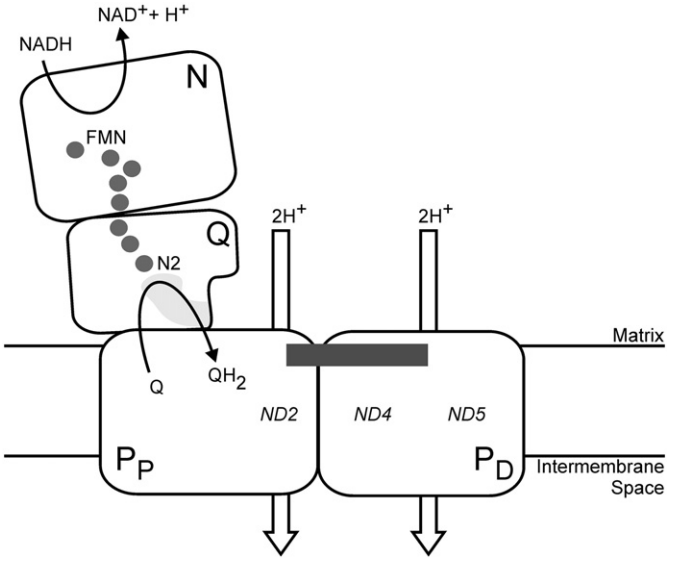

Fig. 1. Functional components and modules of mitochondrial complex I. The scheme shows the four modules of complex I. The N-module transfers hydride from NADH into FMN. Then the electrons are transferred via iron–sulfur clusters (gray dots) into the Q-module, where they reduce ubiquinone (Q) to ubiquinol (QH_2) at a site that resides at the interface of the 49-kDa and the PSST subunit (not shown). This redox reaction is coupled to pumping of 2×2 protons across the proximal (P_P) and the distal (P_D) domain of the membrane integral P-module. The two modules that comprise the Na^+/H^+ antiporter homologous subunits ND2, ND4 and ND5 are connected by a helical transmission element (gray bar, [16]).

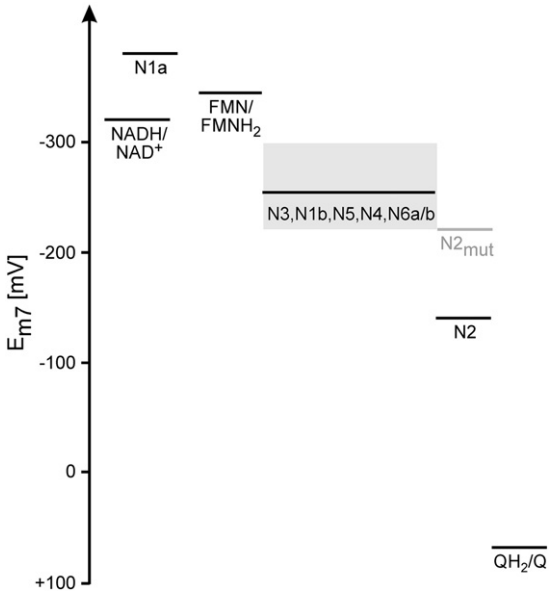

Fig. 2. Redox midpoint potentials of complex I substrates and prosthetic groups. The horizontal bars indicate the approximate redox midpoint potential at pH 7. Six of the iron–sulfur clusters are considered to have midpoint potentials within a rather narrow range indicated by a gray box. The shifted potential of cluster N2 in a mutant ($N2_{mut}$) of a histidine next to it is shown in gray [30]. N1a, N1b, N2, N3, N4, N5, N6a, and N6b, iron–sulfur clusters of complex I.

like subunits are already found in all kinds of membrane bound hydrogenases.

X-ray crystallographic analysis of bacterial [15] and mitochondrial [16] complex I revealed that these functional modules have indeed structural correlates. The membrane integral P-module can be subdivided into a proximal (P_P) and a distal (P_D) module (Fig. 1) that can be dissected both structurally [16] and functionally (Dröse et al., submitted).

3. Iron–sulfur clusters

Bacterial complex I contains up to nine and mitochondrial complex I eight iron–sulfur clusters, two of which are of the binuclear Fe_2S_2 type [22]. The other clusters are of the tetranuclear Fe_4S_4 type. A chain of seven of these clusters connects the FMN at the NADH-oxidation site in the N-module with the quinone reduction site in the Q-module (Fig. 1). Some aspects of the nomenclature and assignment of the iron–sulfur clusters of complex I are still under debate [23–25], but these issues are not addressed here.

Cluster N2, the last iron–sulfur cluster in the chain (Fig. 1), is not only the immediate reductant of quinone, but it also stands out for several other reasons. As shown first by Tomoko Ohnishi [26–29], in many species it exhibits a significantly higher and pH-dependent redox midpoint potential ($E_{m7} \cong -150$ mV) than the other iron–sulfur clusters of complex I ($E_{m7} \sim -250$ mV; Fig. 2). However, the functional implications of these properties remain unclear. Mutagenesis of a histidine forming a hydrogen bond to cluster N2 to a methionine abolished the pH-dependence of the midpoint potential and shifted it to around −210 mV. Unexpectedly, this marked change had no significant effect on electron transfer or proton pumping activity [30].

Considering that even the closest cluster (N2) resides 25–30 Å above the membrane plane in complex I [15,16] it seems not immediately obvious how the redox centers of complex I could be involved in the proton pumping mechanism. Moreover, estimation of the electron transfer rates between the clusters based on their distances suggested that an electron can travel in less than 100 μs from FMN to quinone [31]. This prediction has been confirmed experimentally [32] and seems to suggest that electron transfer in complex I occurs at a much faster timescale than turnover that takes about 5 ms. Indeed during steady-state turnover, the iron–sulfur clusters of complex I are largely reduced [33] indicating that its turnover rate is limited entirely by steps associated with the chemistry of quinone reduction. In other words, the seven iron–sulfur clusters seem to act like an electron buffer poised to efficiently deliver electrons to the site of quinone reduction. An immediate implication of this scenario is that during turnover the immediate electron donor for quinone, iron–sulfur cluster N2 is only transiently oxidized and will always be re-reduced on the microsecond timescale, while quinone chemistry and proton pumping take milliseconds. It follows that structural changes observed when comparing fully reduced and fully oxidized complex I [34,35] are not likely to be relevant for the proton pumping mechanism. Rather, the key for understanding this process seems to lie in the chemistry of quinone reduction.

4. Quinone binding

The number and position of quinone binding sites in complex I have been a controversial issue for a long time. By analogy to the cytochrome bc_1 complex, different classes of hydrophobic inhibitors with different behavior in steady-state inhibition kinetics [36,37] seemed to suggest the presence of several independent quinone binding sites. However, direct competition studies [38] and site directed mutagenesis [39] indicate that these inhibitor classes reflect distinct, but overlapping binding regions in a common binding pocket formed by the central 49-kDa and PSST subunits of the Q-module. The pocket seems to open to the bulk phase near the phospholipid head group region on the negative side of the bioenergetic membrane and extends into the immediate vicinity of iron–sulfur cluster N2 (Fig. 1). This position adjacent to the end of the electron transfer chain within complex I and extensive exploration of the residues at its rather hydrophilic inner surface by site directed mutagenesis [40] indicate that the pocket provides access for the head group of the substrate

quinone to its electron donor. For the mitochondrial enzyme, mutagenesis of a conserved tyrosine in the immediate vicinity of cluster N2 resulted in markedly reduced activities for the endogenous substrate ubiquinone-9 or decylubiquinone and an up to 20-fold increase of the K_m value for ubiquinone-1 suggesting direct interaction of this residue with the substrate [41]. Remarkably, the recent X-ray structural analyses of complex I show that this residue resides about 25 Å above the membrane [16]. On the other hand, mutations in several of the hydrophobic proteins of the P-module that affect the steady-state kinetics [42,43] and binding of photoaffinity derivatives of complex I inhibitors [44–46] point towards an involvement of these subunits in quinone binding. Indeed, subunits ND1 and ND3 that seem to be directly connected to the peripheral arm of complex I [15] may contribute to the access path for quinone to the binding pocket in the Q-module described above. However, since no redox prosthetic group is found within electron tunneling distance to the P-module, these results most likely do not indicate the presence of another quinone active site in the hydrophobic domain. Rather they seem to reflect indirect effects and long range interactions.

Quinone binding in the immediate vicinity of iron–sulfur cluster N2 is also evident from a paramagnetic interaction between a semiquinone intermediate and this redox center that can be observed by EPR spectroscopy in coupled bovine submitochondrial particles during steady-state. This semiquinone is abolished by uncoupler [22,47–49]. Consistent with direct binding to the tyrosine residue discussed above (Fig. 3), a distance of about 10 Å has been calculated from these data between this "fast relaxing" complex I semiquinone species SQ_{Nf} and cluster N2 [22]. A second slow relaxing semiquinone species SQ_{Ns} has been observed in the steady-state of complex I that is not dependent on the presence of a membrane potential. SQ_{Ns} does not couple paramagnetically to reduced cluster N2 [22]. From this it has been concluded that SQ_{Ns} should be a least 20 Å away indicating the binding of a second quinone to complex I. However, the paramagnetic interaction may not only be abolished by increasing the distance between the centers, but also e.g. by transiently introducing water molecules nearby that could markedly change the properties of the interacting spins. Moreover, since the observed intensities of both semiquinones always reflect an occupancy for each species of well below 0.5, the fast and slow relaxing signals could also reflect two positions of the same semiquinone molecule within the binding pocket. The dependence of only SQ_{NF} on the proton motive force could then indicate that these two positions corresponded to two states in the pump cycle of complex I. These observations provide important clues of the catalytic mechanism of complex I, but it has to be stressed that so far semiquinones were only observed in bovine complex I. This probably does not imply a different mechanism in other species, but rather highlights the transient nature of these catalytic intermediates that seem to be observable only under very special circumstances. Furthermore, it should be noted here that although one bound quinone has been found in preparations of bacterial complex I [32], it was also shown that with short chain ubiquinone analogs full activity and proton pumping capacity are observed with quinone-depleted mitochondrial complex I [50]. Thus there is no clear indication for the presence of a tightly bound "prosthetic" ubiquinone.

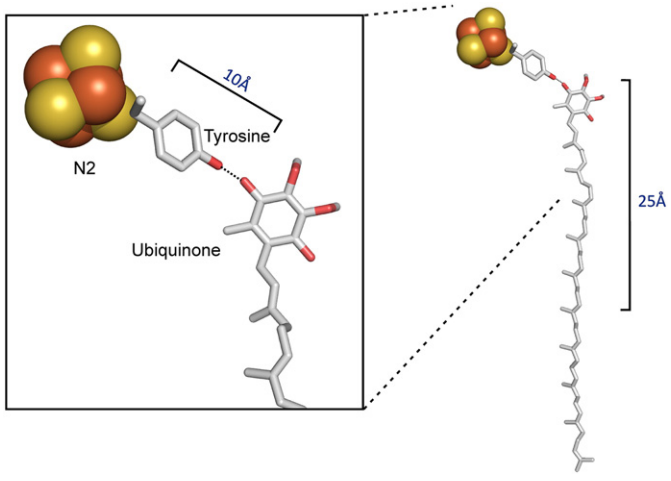

Fig. 3. Binding interaction of ubiquinone near iron–sulfur cluster N2. Ubiquinone is proposed to form a hydrogen bond to a conserved tyrosine close to iron–sulfur cluster N2 (N2). On the right the natural substrate in humans ubiquinone-10 is shown in extended conformation to illustrate how much at least of the side-chain has to leave the membrane domain 25 Å below the conserved tyrosine for the head group to reach its binding site.

Overall there is now good evidence that the active site for the reduction of the quinone substrate of complex I is located within ~10 Å of iron–sulfur cluster N2 and thus about 25 Å above the membrane surface (Fig. 3). For efficient access of the hydrophobic substrate to this unusually positioned active site, complex I should contain some kind of access path that should allow exchange with the ubiquinone pool in the membrane within a few milliseconds. Indeed, there are indications for additional sites interacting with quinone that could be involved in guiding the substrate to its electron donor in the Q-module thereby facilitating its exchange. At any rate, for the general mechanistic discussion presented here, the simplest assumption of a single quinone binding site seems most appropriate. The position of this site, spatially well separated from the membrane domain, renders any direct coupling mechanism rather unlikely. Therefore, one has to conclude that the events associated with the reduction of the quinone must somehow generate directed conformational changes that drive the pumping machinery located in the P-module of complex I.

5. Pump modules

The P-module comprises the seven hydrophobic central subunits of complex I that are encoded by the mitochondrial genome in most eukaryotic species. As mentioned above, the three largest of these subunits, ND2 (NuoN/Nqo14), ND4 (NuoM/Nqo13) and ND5 (NuoL/Nqo12), exhibit homology to Mrp-type Na^+/H^+ antiporters that have retained charged residues within their membrane domain [21,51,52] making them the prime candidates to harbor the pump sites of complex I. ND4 and ND5 reside in the P_D module and ND2 right next to it in the P_P module (Fig. 1). These subunits were proposed to account for the pumping of three of the four protons per NADH oxidized [15]. It should be noted however that conclusions on the function of a protein cannot be drawn on the basis of homology and conservation of specific residues alone. For example, the fact that despite very high structural similarity and the presence of all necessary cofactors, only one branch in most dimeric photoreaction centers exhibits functional electron transfer demonstrates how misleading such conclusions can be [53]. Moreover, if all three antiporter type subunits of complex I pump protons, a fourth pumping device of another type has to be postulated. Indeed there are conserved charged residues in the membrane domain of subunit ND1 (NuoH/Nqo8). However, involving this subunit in proton pumping [52] would mean that the fourth module was not based on the same evolutionary conserved system as the other three. Alternatively, the fourth pump would have to operate by a completely different principle. Obviously, this could be a pumping event linked to the redox reactions of ubiquinone in a direct chemical fashion [14], but this seems rather unlikely considering the rather long distance between its binding site and the membrane through which the protons have to be transported (Fig. 1). Overall, assuming the synchronous operation of four pump sites spread over a distance of more than 100 Å by a single redox event seems not very likely and there is no compelling evidence for this postulate.

On the other hand, the detectable accumulation of semiquinone intermediates during steady-state turnover of complex I indicates an

internal energetic barrier suggesting that not only the first, but also the second electron transfer onto the substrate quinone is linked to proton pumping. For a complete catalytic cycle of four protons pumped upon complete oxidation of one NADH this would suggest two one-electron transfers onto ubiquinone to generate two conformational strokes that result in the pumping of two protons each time. Recent structural [15,16] and functional (Dröse et al. submitted) studies suggest that the P_P and the P_D module of complex I each contain one of the corresponding pump sites and that these sites are connected by a long helical "transmission element" (Fig. 1) that couples the two pumps conformationally in a way reminiscent to the function of a coupling rod of a steam engine [16].

6. Two-state stabilization-change mechanism

Our knowledge about the structure and function of complex is far from being complete and a number of controversial issues remain. However, from the recent X-ray structural work [15,16] we understand the overall architecture of complex I and the identity and arrangement of its functional modules. Structure/function studies can now be designed and interpreted on the basis of this structural framework and provide critical constraints for possible mechanistic schemes. The hypothetical mechanism for the redox driven proton pump of ubiquinone dependent complex I described below is based on the following scenarios.

- Proton pump sites in the P_P and the P_D module of complex I are operated indirectly via long range conformational changes.
- These conformational changes are generated during the two electron redox chemistry of ubiquinone in the substrate binding pocket of the Q-module.
- The iron–sulfur clusters in the N- and Q-module serve together with FMN as reservoir of electrons that keep the redox poise of the system at around −250 mV or less.

It seems quite feasible that introducing a negative charge into the binding pocket when reducing ubiquinone to semiquinone by cluster N2 could induce the conformational change that drives the proton pumps. For example, fully conserved arginines next to cluster N2 have shown to be critical for complex I activity and could thus be involved in mediating this change [40,54,55]. Moreover, the dependence of the semiquinone species SQ_{Nf} on the presence of a proton motive force [47] indicates a direct link between this steady-state intermediate and the proton pumps. The energy for pumping the protons could be provided by stabilizing the anionic semiquinone thereby shifting its midpoint potential by about 200 mV (Fig. 4). The stabilized species would then correspond to SQ_{Ns}. The fact that a semiquinone species can be observed during steady state as such also indicates that there must be an energetic barrier associated with the second electron transfer. This electron transfer results in a negatively charged ubiquinol anion electrostatically quite similar to a semiquinone anion. It could thus induce the same conformational change and then provide a second stroke transmitted to the pump modules. In this case stabilization would result in a shift of the apparent pK value by 3.5 units equivalent to about 200 mV. The apparent midpoint potentials and pK values for the bound ubiquinone intermediates depicted in Fig. 4 can only be considered as crude estimates. They are derived from values published for the compound in 80% ethanol [56,57]. It is assumed that, relative to these values, the environment of the binding pocket shifts the anionic intermediates by about −60 mV in the non-stabilizing state, but by +140 mV in the stabilizing state. As an approximation this accounts for the fact that formation of the first ubiquinone intermediate, the ubisemiquinone should be endergonic to account for the observed redox state of the iron–sulfur clusters.

Anionic charge stabilization provides a rather simple rationale for driving the proton pumps of complex I based on just two conformational

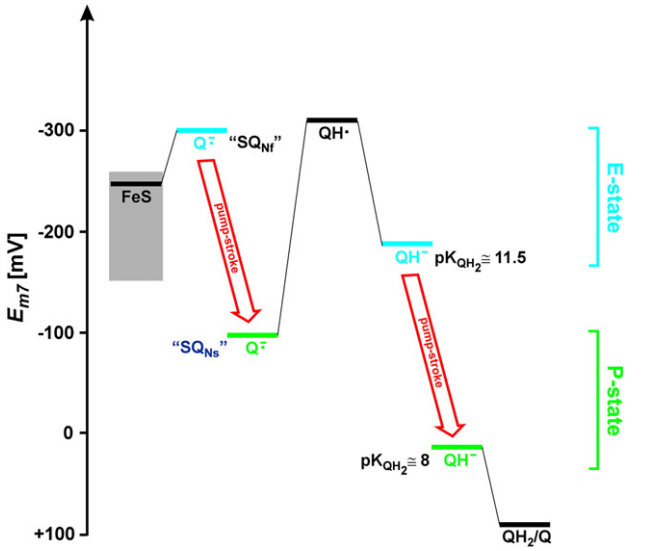

Fig. 4. Estimated redox midpoint potentials of ubiquinone intermediates. The potentials differ by about 200 mV between the E-state (blue) and the P-state (green). This energy change is proposed to provide the driving force for the conformational stroke operating the proton pumps. See text and Table 1 for further details. FeS, reduced iron sulfur clusters; Q, ubiquinone; $Q^{\cdot -}$, ubisemiquinone anion; $QH^{\cdot}$, protonated semiquinone; QH^-, ubiquinol anion; QH_2, ubiquinol.

states that would switch the pump sites between the input and the output state for protons. Since uncontrolled reduction of the semiquinone and uncontrolled protonation of the ubiquinol anion would dissipate the energy required to drive the proton pumps, a strictly controlled access for electrons and protons to the substrate is a critical requirement for a functional pump cycle based on this principle (Fig. 5). It results from these considerations that the two conformational states of complex I should have the following symmetrically opposing properties (Table 1).

- In the E-state (for electron transfer) electron transfer from cluster N2 to ubiquinone or semiquinone occurs at a high rate, but the substrate is shielded from the bulk phase to prevent protonation. In this state anionic species are destabilized and the pump sites are in the input conformation.
- In the P-state (for protonation) electron transfer from cluster N2 to ubiquinone or semiquinone is prevented, but protonation from the

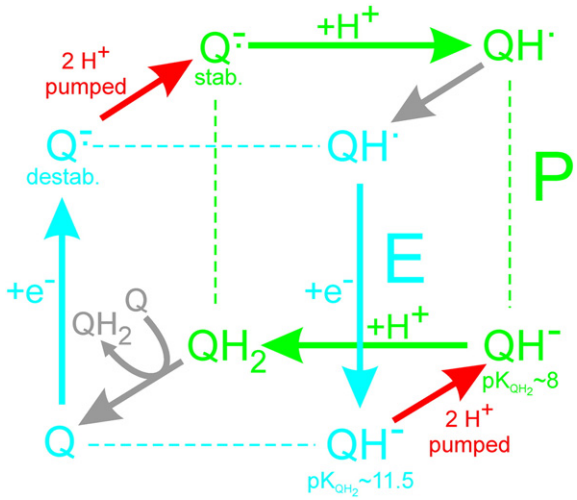

Fig. 5. "Scheme of squares" illustrating the pump cycle through the ubiquinone intermediates. The protons are pumped upon switching from the E-state (blue) to the P-state (green). See text for further details and legend of Fig. 4 for abbreviations.

Table 1
Properties of the two postulated conformational states of complex I.

	E-state	P-state
Stabilization of anionic quinone	No	Yes
Electron transfer cluster N2 → quinone	Yes	No
Proton access to quinone	No	Yes
Mode of pump sites	Input	Output

bulk phase is possible. In this state anionic species are stabilized and the pump sites are in the output conformation.

On one hand the conformational change induced by charge stabilization could move the substrate away from its electron cluster N2 donor in the P-state effectively slowing down electron transfer. On the other hand this rate is also dependent on the packing density between donor and acceptor and the reorganization energy of the reaction. The commonly used "ruler" linking electron transfer uses generic values for these parameters [31]. Indeed Moser and Dutton emphasize that their expression for calculating electron transfer is no longer valid, if bond making and breaking occurs at catalytic sites, because then non-electron tunneling barriers will be relevant as well [31].

The pump cycle for this stabilization change mechanism fitted into the structural framework is illustrated in Fig. 6. Note that this "two stroke" mechanism requires only two pump sites in the P-module to allow for an overall pumping stoichiometry of 4 $H^+/2e^-$. While the structural basis for the transfer of conformational energy from the ubiquinone binding site to the first pump site is still unclear, the recently discovered transmission element [15,16] is likely to be responsible for the transfer from the proximal to the distal pump site. However at this point we can only speculate how and to what extent the components of the pumping machinery have to move during the pump cycle.

Note that tight coupling between the pump sites and the redox chemistry of ubiquinone would drive the system in the presence of a high membrane potential from the P-state into the E-state (Fig. 4). This provides an explanation for the different behavior of the observed semiquinone species SQ_{Ns} and SQ_{Nf} [47,48] that would then in fact reflect these two states. Moreover, together with the markedly lowered pK values for ubiquinol in the P-state this provides a rationale how, based on the same principles, complex I can operate in reverse mode. Full reversibility is thus an inherent feature of the proposed mechanism.

As compared to ubiquinone, only half of the free energy is available to drive the proton pumps when the substrate is menaquinone that has an about 200 mV more negative redox midpoint potential. As evident from the much lower pK of menaquinol, a negative charge in the naphtoquinone ring is more delocalized than in the benzoquinone ring of ubiquinone. This could result in weaker electrostatic interactions and thus a weaker power stroke predicting that in the absence of a membrane potential complex I could pump with the same stoichiometry with both substrates. However, at high membrane potentials complex I could then only function with ubiquinone. Alternatively, the Na^+-antiport activity of complex I recently reported for some menaquinone containing bacteria [58] may abrogate this limitation.

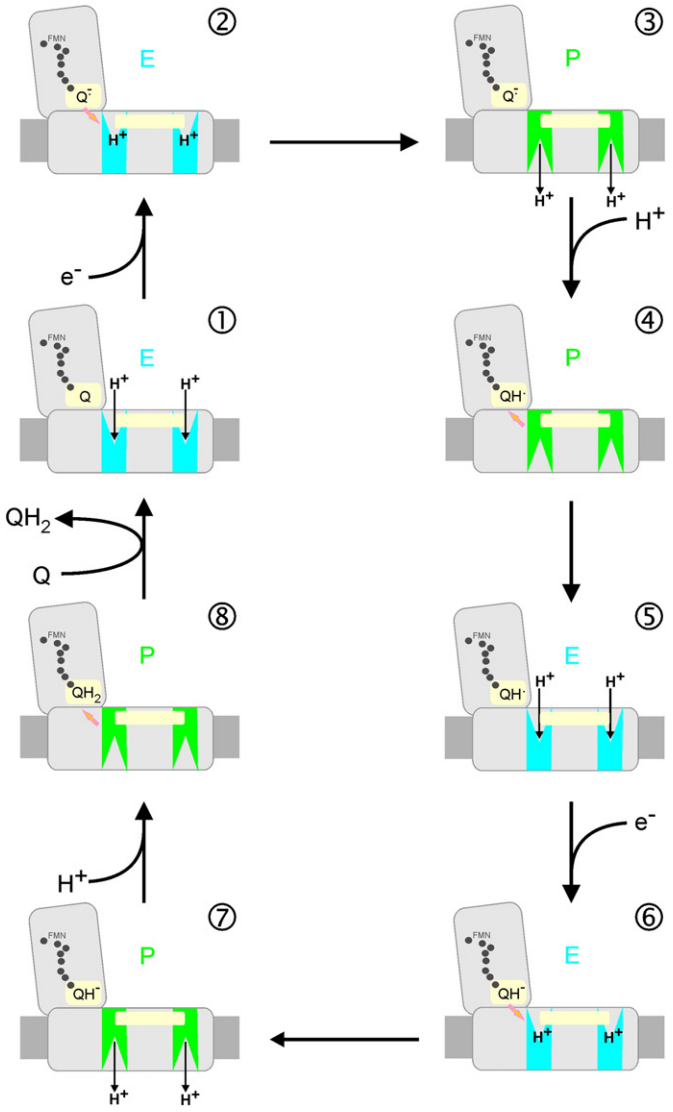

Fig. 6. Pump cycle of the two-state stabilization change mechanism. The redox intermediates of ubiquinone (see Figs. 4 and 5) drive conformational changes in the two pump modules of the membrane arm. The stabilization change upon formation of the anionic intermediates $Q^{\cdot-}$ and QH^- switch the pump sites from the input mode of the E-state (blue) into output mode of the P-state (green). The two pump sites are conformationally linked by the movement of the transmission element (light yellow bar). Note that at this time there is no evidence available to decide whether this is a shift, a rocking motion or a rotation.

7. Conclusion

Clearly, still many different mechanisms of redox-driven proton pumping are conceivable based on the currently available evidence on the structure and function of complex I. However, the two-state stabilization-change mechanism proposed here seems attractive, since it is based on a rather simple set of principles and inherently provides an explanation for the forward and reverse mode of complex I. Testing the principles and specific predictions of the mechanism proposed here will guide the way to prove or disprove it and to further develop our mechanistic understanding of complex I. To this end, apart from the urgent need to obtain structural information for complex I at higher resolution, determining the conformational changes during turnover and the biophysical properties of the ubiquinone intermediates will be of critical importance.

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